

<b>(51) International Patent Classification 7 :</b> <b>C12Q 1/68, G01N 33/543</b>	<b>A1</b>	<b>(11) International Publication Number:</b> WO 00/65097  <b>(43) International Publication Date:</b> 2 November 2000 (02.11.00)	
<b>(21) International Application Number:</b> PCT/US00/11282  <b>(22) International Filing Date:</b> 26 April 2000 (26.04.00)		<b>(81) Designated States:</b> AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
<b>(30) Priority Data:</b> 09/299,831                  26 April 1999 (26.04.99)                  US			
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<b>(54) Title:</b> BIOCHIP AND METHOD FOR MAKING IT			
<b>(57) Abstract</b>			
<p>Methods for preparing a hydrogel biochip wherein a plurality of biomolecular probes are bound to a hydrogel prepolymer either prior to or simultaneously with polymerization of the prepolymer. While either hydrogel is polymerizing, it is microspotted onto a solid substrate to which the hydrogel becomes covalently bound in the form of a hydrogel microdroplet. Adjustment of the reactivity of the prepolymer and the polymerization conditions provides effective control of the density of biomolecular probe immobilization. Resulting biochips containing a plurality of such microdroplets having different biomolecules bound thereto are useful for gene discovery, gene characterization, functional gene analysis and related studies.</p>			
$\text{O}=\text{C}-\text{N}-(\text{X})_n-\text{N}=\text{C}=\text{O} + \text{Y}-\text{NH}_2 \xrightarrow{\text{Organic solvent}} \begin{array}{c} \text{O} \\   \\ \text{Y}-\text{N}-\text{C}-\text{N}-(\text{X})_n-\text{N}=\text{C}=\text{O} \\   \\ \text{H} \end{array}$ <p>X = Polyethyleneoxide or copolymer of polyethyleneoxide and polypropyleneoxide capped with polyisocyanates and lightly cross-linked with polyols</p> <p>Y = Organic solvent soluble biomolecules</p> $\downarrow \begin{matrix} \text{Polymerization} \\ \text{H}_2\text{O} \end{matrix}$ $\left[ \begin{array}{c} \text{Y}-\text{N}-\text{C}(=\text{O})-\text{N}-(\text{X})_n-\text{NH}_2 \\   \\ \text{O}=\text{C}-\text{N}-(\text{X})_n-\text{N}=\text{C}=\text{O} \end{array} + \text{CO}_2 \uparrow \right]$ $\longleftarrow \begin{array}{c} \text{Y}-\text{N}-\text{C}(=\text{O})-\text{N}-(\text{X})_n-\text{NH}_2 \\   \\ \text{O}=\text{C}-\text{N}-(\text{X})_n-\text{N}=\text{C}=\text{O} \end{array}$ <p>Continuation of polymerization until all isocyanates are consumed.</p>			

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## BIOCHIP AND METHOD FOR MAKING IT

This application is a continuation-in-part of U.S. Serial No. 09/299,831, filed April 26, 1999, the disclosure of which is incorporated herein by reference.

The present invention relates to new methods of making biochips and the biochips resulting therefrom. In particular, the new method described herein provides for rapid, simple and cost effective construction of biochips by employing isocyanate-functional hydrogels to immobilize biomolecular probes on the substrate. In particular, both organic-solvent-soluble biomolecules, like peptide nucleic acids (PNAs), and water-soluble biomolecules, like DNA, RNA and other oligonucleotides, are easily and efficiently bound to a hydrophilic polymer either before or during polymerization thereof. In addition to the improved method of making the biochips described herein, the biochips themselves have improved characteristics including superior stability providing a much improved shelf-life and greater flexibility in use. Such biochips are useful for gene discovery, gene characterization, functional gene studies, screening for biological activity and related studies.

**BACKGROUND OF THE INVENTION**

Agents that selectively bind to DNA, RNA or analogs, such as peptide nucleic acids (PNAs) are of significant interest to molecular biology and medicinal chemistry as they may be developed into gene-targeted drugs for diagnostic and therapeutic applications and may be used as tools for sequence-specific modification of DNA. Additionally, such reagents may be used as tools for determining gene sequences and for functional gene analyses.

Until recently, the processes of gene discovery, characterization and functional gene analysis have been difficult and costly and have required tremendous amounts of time. However, within about the last ten years, methods of isolating arrays of biomolecules on various supports, referred to as biochips, have been developed and have been employed in DNA synthesis, sequencing, mutation studies, gene expression analysis and gene discovery. Generally the biochips are micromatrices (i.e., microarrays) of biomolecules bound to a substrate, either directly or through a linking group or, more recently, by way of a gel layer. Most biochips are designed to facilitate synthesis of biomolecules at known locations on a substrate. For example, one such biochip employs light and a series of photo-lithographic masks to activate specific sites on a substrate, such as glass, in order to selectively bind nucleic acids thereto and, subsequently, to attach additional nucleic acids to form known oligonucleotides at the desired locations. This process of using light and photolithographic masks to activate specific sites on a substrate is similar to the processes used in production of the microelectronic semiconductor chip.

Unfortunately, these first generation biochips are very expensive to produce, requiring large capital investments, process engineering and equipment. Furthermore, this synthesis method of forming oligonucleotides in a single layer on a substrate results in a low sensitivity biochip requiring an expensive laser confocal fluorescence microscope for adequate detection of DNA specifically hybridized to the chip. U.S. Patent No. 5,744,305, issued to Fodor, et al. (hereinafter, the '305 patent), provides an example of the use of photolabile protecting groups and photolithography to create arrays of materials attached to a substrate, describing a synthetic strategy for the creation of large

scale chemical diversity wherein a substrate, such as glass, is derivatized, either directly or by addition of a linker molecule, to include an amino group blocked with a photolabile protecting group. Masking techniques are employed to permit selective deprotection of the photolabile groups within a specified, known location on the substrate. The deprotected region is then reacted with a "building block" molecule, for example an oligonucleotide, also containing a photolabile protecting group, such that the building block molecule is covalently bound to the active group at the surface of the substrate (or linker). This process is then repeated using the masks to direct synthesis of polymers, for example biomolecules, such as oligonucleotides or peptides, of specific sequences at predefined locations on the substrate.

The synthetic strategies described in the '305 patent contemplate providing from about 10 to about  $10^8$  different sequences on a single substrate. Additionally, it is stated that the predefined regions on the substrate, wherein individual polymers are to be synthesized, are from about  $10^{-10}$  cm<sup>2</sup> to about 1 cm<sup>2</sup>. While the examples presented in the '305 patent primarily involve synthesis of peptides or nucleotides, it is stated that the same techniques may also be employed in the synthesis of other polymers. Similarly, various linker groups, preferably inactive or inert, for linking the synthesized polymer to the substrate are discussed in the '305 patent, as are various protecting groups for protecting an active site on the monomers which protecting groups may be selectively removed for directed synthesis of the polymer. Also discussed in some detail in the '305 patent is a binary masking technique utilized in one embodiment for directed synthesis of the array. Unfortunately, the strategies described in the '305 patent suffer from many of the same disadvantages as

other prior art methods and apparatus. The arrays are expensive to manufacture and use, require multiple steps and lengthy incubation/washing times during manufacture and, significantly, permit synthesis in only a single  
5 layer.

In view of the low sensitivity of these first generation biochips, second generation biochips have been developed.

One example of a second generation biochip is  
10 described in U.S. Patent Nos. 5,736,257 and 5,847,019, issued to Conrad, et al. The '257 and '019 patents describe a process of synthesizing a biochip comprising providing a substrate, such as glass, having surface hydroxyl groups; reacting the substrate surface hydroxyl  
15 groups with silanes to bind a molecular layer of vinyl groups upon the substrate; placing an acrylamide compound on the molecular layer, which acrylamide compound can participate in a free radical polymerization reaction to make a polymerized network layer bound to the molecular  
20 layer; photo-activating the polymerized network layer to make a patterned photo-activated polymerized network; and placing upon the photo-activated polymerized network layer, for example by synthesis thereon, one or more similar or dissimilar biomolecules to bind to the  
25 patterned photo-activated polymerized network layer.

The biochips disclosed in the '257 and '019 patents are somewhat similar to the first generation biochips of Fodor, et al. in that they employ photolithographic techniques to direct binding (or synthesis) of  
30 biomolecules to an array. However, in contrast to the first generation biochips, the biochips of the '257 and '019 patents employ a polyacrylamide network on top of a molecular layer of vinyl groups, thereby giving a third dimension to the gel cells. Still, as will be readily  
35 appreciated by those of skill in the art, production of biochips in accordance with the disclosures of the '257

and '019 patents is not only expensive but also time-consuming.

U.S. Patent No. 5,552,270, issued to Khrapko, et al., describes a method of sequencing DNA which utilizes  
5 a second generation biochip comprising a solid support and a matrix that includes an array of oligonucleotides of desired lengths. The matrix is attached to the support by means of a gel layer having a thickness of 30  $\mu\text{m}$  or less; a gel layer is described in the form of a set  
10 of spaced apart square "dots". In contrast to the single layer format described in the '305 patent, the gel layer of the '270 patent provides for a three-dimensional attachment of oligonucleotides to the substrate at a capacity therefore exceeding the capacity of the mono-  
15 molecular layer of the first generation biochips. This second generation biochip polymerizes a polyacrylamide gel between two glass slides spaced about 30  $\mu\text{m}$  apart. One slide is removed, and the gel-coated lower slide is dried. Portions of the gel are then mechanically removed  
20 to leave the spaced dots. In alternative embodiments which also employ polyacrylamide gel matrices as described in U.S. Patent Nos. 5,741,700, 5,770,721 and 5,756,050, issued to Ershov, et al., gel parts are removed from the slide using a laser.

25 Although the polyacrylamide gel matrices described in the Ershov, et al. patents and the '270 Khrapko, et al. patent are hydrogels having a water content, at equilibrium, of about 95% to 97%, and provide favorable diffuseability for target molecules such as DNA's, they  
30 have significant disadvantages. A significant disadvantage to such second generation biochips is their cost. The polyacrylamide-based biochip described by Ershov, et al., is based upon the polymerization of acrylamide monomers by a free radical initiation or  
35 ultraviolet radiation process; however, this polyacrylamide-based gel biochip is constructed in a

multi-step process that is lengthy and labor-intensive. Production of such a biochip requires cumbersome multi-step processing including polymerization and binding to the surface of the glass substrate; mechanical or laser  
5 cutting to form micro-squares of gel matrix on the substrate; activation step using hydrazines; and finally reaction with the oligonucleotides. Due to the polymerization process of inherent polyacrylamide gels, these steps must be performed independently. Thus, the  
10 total time required to produce a single biochip by such methods is at least about 24 to 48 hours. Furthermore, after each step, thorough washings and/or other special cares must be taken before the next step is begun. For example, the oligonucleotide derivatization step requires  
15 a long incubation period, such as twenty-four to forty-eight hours.

Still another significant disadvantage to such second generation biochips lies in the fact that the reaction of the oligonucleotides with the hydrazine  
20 groups forms unstable morpholine derivatives resulting in a very short shelf half-life for the biochip of approximately thirty-six hours at room temperature. Thus, there is a significant need in the industry for a simple, cost effective, rapid method for constructing a  
25 reliable multi-functional biochip having high sensitivity and a reasonably long shelf-life that may be used in gene discovery, gene characterization, functional gene analysis and related studies.

#### **SUMMARY OF THE INVENTION**

30 The present invention provides a rapid, simple, cost effective method for construction of an improved biochip and an improved biochip resulting therefrom. For example, the method described herein allows biomolecular probes to be bound prior to or simultaneously with  
35 polymerization of the gel, thereby permitting a simple,



one or two step process for production of the biochip. Thus, what has heretofore required multiple steps, multiple washings, and lengthy reaction times, can now be performed in essentially one or two steps. Further  
5 provided herein are improved biochips having increased sensitivity, superior stability, both in use and with respect to shelf-life, and improved cost effectiveness.

Such biochips can be formed by dispensing optically transparent microdroplets of hydrogel prepolymer having  
10 biomolecular probes suitably, e.g. covalently, attached thereto onto a substrate. It may however be preferable to prepare isocyanate-functional hydrogel biochips having a biomolecule immobilized thereon, by providing an organic solvent solution of an isocyanate-functional  
15 hydrogel prepolymer; providing a buffered aqueous solution of the biomolecule to be immobilized thereon; mixing the two solutions to covalently bind the biomolecule to the hydrogel prepolymer while initiating polymerization; and then dispensing the polymerizing  
20 hydrogel prepolymer onto a solid substrate so that the polymerized hydrogel becomes suitably bound to such substrate. In certain preferred embodiments, the hydrogel has sufficient active isocyanate groups thereon to both participate in the covalent binding of  
25 biomolecular probes to the hydrogel prepolymer as well as participate in polymerization of the hydrogel.

In one aspect, the invention provides a method of preparing a hydrogel biochip having a biomolecule immobilized thereon, the method comprising the steps of  
30 (a) providing an organic solvent solution of isocyanate-functional hydrogel prepolymer; (b) providing a solution of a biomolecule; (c) covalently binding the biomolecule to the hydrogel prepolymer; and (d) initiating polymerization of the hydrogel prepolymer.

In another aspect, the invention provides a method of preparing a biochip having an organic solvent soluble biomolecule covalently bound thereto, the method comprising (a) providing a solid substrate having a top surface; (b) providing a solution comprising the biomolecule in an aprotic organic solvent; (c) providing an isocyanate-functional hydrogel prepolymer in an aprotic organic solvent; (d) derivatizing the prepolymer with the biomolecule from step (b); (e) initiating polymerization of the derivatized hydrogel prepolymer of step (d) by adding a buffered aqueous solution thereto; and (f) microspotting the polymerizing hydrogel solution of step (e) onto the top surface of the substrate of step (a), resulting in attachment of the hydrogel and immobilized biomolecule to the substrate.

In yet another aspect, the invention provides a biochip comprising (a) a solid substrate having a top surface; (b) a plurality of hydrogel cells covalently bound to the top surface of the substrate, wherein each hydrogel cell is formed of an isocyanate-functional polymer; and (c) a biomolecular probe covalently bound to and within at least one of the hydrogel cells. Preferably, a different biomolecular probe will be bound within each hydrogel cell, and preferably the covalent binding is via the isocyanate groups.

In a further aspect, the invention provides a biochip comprising (a) a solid substrate having a top surface; (b) a plurality of hydrogel cells covalently bound to the top surface of the substrate, wherein the hydrogel comprises polyethylene glycol, polypropylene glycol, or copolymers thereof; and (c) different biomolecular probes which are covalently bonded to and within different hydrogel cells.

In a still further aspect, the invention provides a biochip comprising (a) a solid substrate having a top surface; (b) a plurality of hydrogel cells covalently

bound to the top surface of the substrate wherein the hydrogel comprises a polymer with urethane-urea linkages; and (c) different biomolecular probes which are covalently bound to and within different hydrogel cells.

5 In a yet further aspect, the invention provides a hybridization assay comprising (a) providing a biochip which comprises a substrate having at least two hydrogel cells bound thereto, each cell having a thickness of at least about 20  $\mu\text{m}$  and comprising a major portion of a  
10 polymer selected from the group consisting of polyethylene glycol, polypropylene glycol and copolymers thereof, wherein at least one hydrogel cell further includes a biomolecular probe covalently bound thereto; (b) contacting the biochip with an analyte solution,  
15 containing a target biomolecule, under hybridization conditions; (c) washing the hydrogel biochip under conditions that remove non-specifically bound and unbound target biomolecule; and (d) detecting the bound target biomolecule.

20 In an additional aspect, the invention provides a hybridization assay comprising (a) providing an organic solvent solution of a hydrogel prepolymer having an active isocyanate content of less than about 1 meq/g; (b) providing a solution of a biomolecule; (c) covalently  
25 binding the biomolecule to at least a portion of the hydrogel prepolymer; (d) initiating polymerization of the hydrogel prepolymer; (e) dispensing the polymerizing hydrogel prepolymer onto a solid substrate such that said solution is covalently bound to the substrate at a  
30 thickness of at least about 20  $\mu\text{m}$ , thereby resulting in a biochip; (f) washing the biochip from step (e) with washing buffer such that remaining active sites within the hydrogel are blocked; (g) contacting the washed biochip with an analyte solution, containing a target  
35 biomolecule, under hybridization conditions; (h) then washing the biochip from step (g) with a second washing

buffer such that non-specifically bound and unbound target biomolecule is removed; and (i) detecting the target biomolecule bound to the hydrogel chip.

Preferably each microdroplet of hydrogel on a biochip contains a different biomolecular probe, thereby permitting the screening of large numbers of biomolecular probes as a part of a single hybridization assay. In one preferred embodiment, the biomolecular probes used in constructing the biochips are PNA probes which provide superior screening functions as compared to traditional DNA and/or RNA oligonucleotides. Alternatively, DNA, RNA or other oligonucleotide probes are used in conjunction with a polyurethane-based or other isocyanate-functional gel matrix.

#### 15 BRIEF DESCRIPTION OF FIGURES

FIG. 1 is a schematic illustrating the reaction of a hydrogel prepolymer with an organic-soluble biomolecule followed by polymerization of the hydrogel, as a part of a process embodying various features of the present invention.

FIG. 2 is a schematic illustrating the alternative reaction of a hydrogel prepolymer with a water-soluble biomolecule, such as DNA, during polymerization of the hydrogel.

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#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In order to provide a three-dimensional gel matrix useful in producing a biochip, the polymer chosen to comprise the gel matrix should preferably have a number of desirable properties, such as: 1) adequate pore size and high water content to permit diffusion of molecules in and out of the matrix; 2) an ability to bind to the surface of a substrate, such as glass; 3) sufficient transparency, in its fully polymerized state, to reduce optical interference with fluorescent tags; 4) sufficient

structural integrity, when fully polymerized, to withstand the forces encountered during use; and 5) adequate shelf life for normal research and clinical use. Furthermore, the selected gel should preferably be easy to produce and use.

Hydrogels are a class of polymers that meet these criteria. Hydrogels are hydrophilic network polymers which are glassy in the dehydrated state and swollen in the presence of water forming an elastic gel.

10 In contrast to the polyacrylamide gel systems of Ershov, et al. and Khrapko, et al., it has now been discovered that isocyanate-functional hydrogels lack most of the disadvantages of the polyacrylamide-based gels while possessing a number of important advantages over 15 the prior art. Isocyanate-functional hydrogel polymers are well known and have been used extensively in the production of absorbent materials such as surgical dressings, diapers, bed pads, catamenials, and the like.

By isocyanate-functional hydrogels are meant organic 20 polymers that are capped with isocyanate groups that function to carry out the desired polymerization and may also covalently bind the biomolecules of interest. For example, they may be polyurethanes formed by reaction between diisocyanates and polyether or polyester polyols.

25 The prepolymers used as a starting material for these isocyanate-functional hydrogels preferably provide hydrated polyurethane, polyurea-urethane and/or polyurea polymer gels. Polyurethane polymers are well known in the art. Hydrogel polymers have been prepared from 30 various prepolymers and used for a wide variety of applications. Typically, hydrogels are formed by polymerizing a hydrophilic monomer in an aqueous solution under conditions such that the prepolymer becomes cross-linked, forming a three-dimensional polymeric network 35 which gels the solution in concentrated form. Polyurethane hydrogels are formed by polymerization of

isocyanate-end-capped prepolymers to create urea and urethane linkages.

The isocyanate-functional prepolymers are often prepared from relatively high molecular weight polyoxyalkylene diols or polyols which are reacted with difunctional or polyfunctional isocyanate compounds. The preferred prepolymers made from polyoxyalkylene diols or polyols that comprise homopolymers of ethylene oxide units or block or random copolymers containing mixtures of ethylene oxide units and propylene oxide or butylene oxide units. In the case of block or random copolymers, at least 75% of the units should be ethylene oxide units. The polyoxyalkylene diol or polyol molecular weight is preferably from 2,000 to 30,000, more preferably from 5,000 to 30,000. Suitable prepolymers may be prepared by reacting selected polyoxyalkylene diols or polyols with polyisocyanate at an isocyanate-to-hydroxyl ratio of about 1.2 to about 2.2 so that essentially all of the hydroxyl groups are capped with polyisocyanate. The isocyanate-functional prepolymer preferably used in this invention contains active isocyanates in an amount of about 0.1 meq/g to about 1 meq/g, preferably about 0.2 meq/g to about 0.8 meq/g. In general, a low molecular weight prepolymer, e.g. less than 2000, should contain a relatively high isocyanate content (about 1 meq/g or higher). The polymerization rate of some of these prepolymers may be very difficult to control and may result in too fast polymerization to effectively microspot. Also, these prepolymers having such high isocyanate content may leave a relatively high content of free amines after polymerization, whose positive charges can increase non-specific binding with negatively charged target DNA samples, resulting in high background signals. Thus, high molecular weight prepolymers containing a relatively low isocyanate content are preferred for certain applications.

Such high molecular weight prepolymers are often prepared by either of two general methods, but others can also be used:

(1) High molecular weight polyol (triol or higher, 5 molecular weight at least 2000) is reacted with polyisocyanate such as isophorone diisocyanate; and

(2) High molecular weight diol (molecular weight at least 2000) is reacted with polyisocyanate and a cross-linking agent such as glycerol, trimethylolpropane, 10 trimethylolethane, triethanolamine or organic triamine.

Aromatic, aliphatic or cycloaliphatic polyisocyanates may be used. The high molecular weight aliphatic isocyanate-capped prepolymers typically gel to a hydrated polymer state in about 20 to 90 minutes, 15 whereas prepolymers capped with aromatic polyisocyanates gel much more rapidly. Examples of suitable di- and polyfunctional isocyanates are as follows: toluene-2,4-diisocyanate, toluene-2,6-diisocyanate, isophorone diisocyanate, ethylene diisocyanate, ethylidene 20 diisocyanate, propylene-1,2-diisocyanate, cyclobexylene-1,2-diisocyanate, cyclohexylene-1,4-diisocyanate, m-phenylene diisocyanate, 3,3"-diphenyl-4,4"-biphenylene diisocyanate, 1,6-hexamethylene diisocyanate, 1,4-tetramethylene diisocyanate, 1,10-decamethylene 25 diisocyanate, cumene-2,4-diisocyanate, 1,5-naphthalene diisocyanate, methylene dicyclohexyl diisocyanate, 1,4-cyclohexylene diisocyanate, p-tetramethyl xylylene diisocyanate, p-phenylene diisocyanate, 4-methoxy-1,3-phenylene diisocyanate, 4-chloro-1,3-phenylene 30 diisocyanate, 4-bromo-1,3-phenylene diisocyanate, 4-ethoxyl-1,3-phenylene diisocyanate, 2,4-dimethyl-1,3-phenylene diisocyanate, 2,4-dimethyl-1,3-phenylene diisocyanate, 5,6-dimethyl-1,3-phenylene diisocyanate, 1,4-diisocyanatodiphenylether, 4,4'-diisocyanatodi- 35 phenylether, benzidine diisocyanate, 4,6-dimethyl-1,3-phenylene diisocyanate, 9,10-anthracene diisocyanate,

- 4,4'-diisocyanatodi-benzyl, 3,3'-dimethyl-4,4'-  
diisocyanatodiphenylmethane, 1,6-dimethyl-4,4'-  
diisocyanatodiphenyl, 2,4-diisocyanatostibene, 3,3'-  
dimethoxy-4,4'-diisocyanatodiphenyl, 1,4-  
5 antracenediisocyanate, 2,5-fluoronediiisocyanate, 1,8-  
naphthalene diisocyanate, 2,6-diisocyanatobenzluran,  
2,4,6-toluene triisocyanate, p,p',p"-triphenylmethane  
triisocyanate, trifunctional trimer (isocyanurate) of  
isophorone diisocyanate, trifunctional biuret of  
10 hexamethylene diisocyanate, trifunctional trimer  
(isocyanurate) of hexamethylene diisocyanate, polymeric  
4,4'-diphenylmethane diisocyanate, xylylene diisocyanate  
and m-tetramethyl xylylene diisocyanate.

- Capping of the selected diols or polyols with  
15 polyisocyanates to form prepolymers may be effected using  
stoichiometric amounts of reactants. The isocyanate-to-  
hydroxyl group ratio may vary as known in this art but  
should preferably be about 1 to about 3, and more  
preferably about 1.2 to about 2.2. The capping reaction  
20 may be carried out using any suitable method or  
procedure, such as at about 20° to about 150°C, under dry  
nitrogen, for about 2 hours to about 14 days, preferably  
in the absence of a catalyst. The preferred temperature  
is about 60° to 100°C. The reaction terminates when the  
25 isocyanate concentration approximates theoretical values.

- Preferred prepolymers include polyethylene glycol  
end-capped with toluene diisocyanate, toluene  
diisocyanate and copolymer of ethylene oxide and  
propylene oxide optionally with trimethylolpropane,  
30 toluene diisocyanate-polyethylene glycol-  
trimethylopropane, methylene diisocyanate-methylene  
homopolymer, polymeric methylene diisocyanate-  
polyethylene glycol, isophorone diisocyanate and polymer  
of ethylene oxide-propylene oxide-trimethylolpropane, and  
35 toluene diisocyanate polyethylene glycol trilactate.  
Such prepolymers are available from Hampshire Chemical



Corp. of Lexington, Mass. as HYPOL PreMA®, HYPOL® 2000, HYPOL® 3000, HYPOL® 4000 and HYPOL® 5000 and include copolymers of polyethylene oxide and a minor amount of polypropylene oxide.

5 All things considered, the main chain of the hydrogel polymer is preferably comprised of polyethylene glycol, polypropylene glycol, or copolymers of polyethylene glycol and polypropylene glycol. While not to be constrained by any theoretical mechanism, it is  
10 believed that the non-ionic, hydrophilic properties of polyethylene glycol and polypropylene glycol hydrogels provide for both low levels of non-specific binding of analyte to the hydrogel and for good compatibility with the immobilized biomolecules so as to maintain native  
15 conformation and bioreactivity thereof. Isocyanate-functional hydrogels advantageously absorb large quantities of liquid quickly and in a relatively uniform manner such that the basic overall shape of the gel material is maintained. Further, the moisture absorbed  
20 by these materials is retained in the absorbent material even under an applied pressure. Such isocyanate-functional hydrogels, e.g. polyurethane-based hydrogels are described in U.S. Patent Nos. 3,939,123 (Mathews, et al.) and 4,110,286 (Vandegaer, et al.). These  
25 polyurethane-based hydrogels have been extensively used as surface coatings and to form flexible or rigid foams, but not to form a three-dimensional matrix to which attach chemical compounds of interest.

In a preferred embodiment, biochips are made using  
30 an isocyanate-functional hydrogel based on a diol or triol of high molecular weight polyethylene oxide, polypropylene oxide, or a copolymer of polyethylene oxide and polypropylene oxide, capped with water-active diisocyanates, and optionally lightly cross-linked with a  
35 suitable cross-linker, such that the quantity of active isocyanates present is predictable, for example

preferably not greater than about 0.8 meq/g. Generally preferred diisocyanates include aromatic-based diisocyanates, such as toluene diisocyanate or methylene diphenyl-isocyanate, as well as aliphatic diisocyanates, such as isophorone diisocyanate. The polymerization of the prepolymer, which may be preformulated in a water-miscible organic solvent, takes place by the formation of urea linkages which occur simply upon the addition of water. This is a distinct advantage over the hydrogel-based biochips previously known, wherein ultraviolet radiation or similarly severe reaction conditions are necessary to initiate polymerization. Not only is the water-activated polymerization system of the present invention safer, less expensive and easier to control, but it allows for derivatization of the prepolymer with the appropriate biomolecular probe either prior to or simultaneously with polymerization.

In one embodiment described herein, the hydrogel is derivatized prior to polymerization with biomolecules soluble in an organic solvent using a simple two to three-minute reaction between the biomolecules and the isocyanates of the prepolymer. By organic-soluble biomolecules are meant, molecules such as PNAs which have been previously derivatized with amine and which are naturally soluble in organic solvents and those which are modified to be so soluble. In order to prevent premature polymerization of the hydrogel in the present embodiment, the derivatization reaction is carried out in an aprotic water-miscible organic solvent such as, for example, dimethylformamide (DMF), N-methyl-2-pyrrolidinone (NMP), acetone, acetonitrile or the like or mixtures thereof. Thus, prior to swelling of the hydrogel or dispensing of the hydrogel onto the substrate, biomolecular probes are covalently bound to the polyurethane-based prepolymer gel. Following such derivatization, the addition of water initiates polymerization, resulting in

biomolecular-derivatized hydrogels, for example, PNA-derivatized hydrogels.

Such use and presence of aprotic solvent in the derivatization of the hydrogel has a number of advantages. First, it helps generate a homogeneous solution of the prepolymer in water. Second, it serves to separate the derivatization step from the polymerization step, whereby controlled yield of biomolecule derivatization to the hydrogel can be achieved. Third, it serves to slow down the generation of carbon dioxide during the polymerization step and effervesce carbon dioxide efficiently by lowering the viscosity of the polymerizing mixture. In the polymerization of the polyurethane-based hydrogels, which in some instances are preferred, carbon dioxide is generated by the reaction of water with the isocyanate groups of the hydrogel prepolymer. This reaction scheme is illustrated, for example in FIGS. 1 and 2, and controlling the generation of carbon dioxide and its escape from the gel may be quite important when a biochip is prepared from such a prepolymer. If polymerization occurs too quickly in a highly viscous mixture, the carbon dioxide generated thereby is not able to escape and becomes trapped within the gel resulting in a discrete foam matrix which may be a problem for continuum of the gel matrix may be important in biochips in order to assure accurate and efficient detection of fluorescence, which is indicative of successful hybridization. The generation of carbon dioxide is controlled by reducing the overall reactivity of water/hydroxide through adding aprotic solvent and by using a relatively low basic pH buffer (pH about 7 to about 8.4), which provides close control of the reaction rate.

A fourth and final advantage to the use of an aprotic solvent to derivatize the hydrogel is the enhancement of the optical transparency of the hydrogel

by reducing any precipitation of the prepolymer. It has been discovered that the ratio of aprotic solvent to water should be at least about 0.25 to 1 and preferably higher, e.g. 0.3 - 0.35 to 1, in order to allow desirably

5 slow polymerization of the gel and, therefore, slow generation of CO<sub>2</sub>, which results in a continuous and transparent gel matrix. Derivatization and polymerization of the hydrogel is generally accomplished in about thirty minutes, which is in stark contrast to

10 the twenty-four to forty-eight hours required for preparation of polyacrylamide-based biochips. Furthermore, the quantity of biomolecules, e.g. PNAs, bound to the prepolymer may easily be adjusted by simply varying the amount of biomolecule added to the reaction

15 (for example, where PNA is the biomolecule to be bound to the gel, from about 10 fmol up to about 1 pmol of PNA may be used for each microdroplet), thereby permitting greater control over the concentration of biomolecule probes within each hydrogel microdroplet.

20 When the hydrogel is derivatized with PNA then deposited onto the solid substrate, after initiation and before completion of polymerization thereof, such may be accomplished by any convenient method, for example by use of a conventional microspotting machine which preferably

25 deposits gel to form an array of microdroplets. While the gel may inherently attach non-covalently to some substrates, the substrate surface is generally derivatized prior to addition of the hydrogel to achieve maximal attachment of the gel to the substrate. For

30 example, in one preferred embodiment where glass is used as the substrate, the glass is derivatized with amine prior to deposit of the polymerizing hydrogel onto its surface. Thus, the polymerizing hydrogel, derivatized with biomolecular probes such as PNAs or DNAs binds

35 strongly to the substrate when it is deposited onto the derivatized glass substrate, via reaction of active isocyanate groups within the prepolymer with the amines

located on the surface of the glass thereby providing covalent attachment of the hydrogel to the substrate.

The biochip substrate may consist of a variety of materials and formats which are conducive to automated  
5 handling during use in a binding assay and later detection of molecules binding to the individual biochip cells. For example, an optically transparent substrate, such as glass or polystyrene, will allow for transmission light detection through the cells and is convenient for  
10 detection modalities using fluorescence or optical absorption. Due to the high binding capacity of three-dimensional hydrogel cells, reflective optical methods are also possible and allow the use of opaque substrates. The use of rigid substrates allows for precision of  
15 alignment during the detection phase of the biochip, but it may not be necessary if proper alignment is incorporated into the cells to facilitate detection. An example would be a flexible format, such as a tape or filament, which could be precisely detected in a scanning  
20 fashion similar to the use of magnetic tape. While optical methods and suitable substrates are preferred due to simplicity, other detection methods used in biochemistry could be used, including the detection of radioactive agents.

25 Advantageously, reactions involved in this system, namely (1) the derivatization of hydrogel prepolymer with the biomolecular probe, (2) the polymerization of hydrogel and (3) the binding of derivatized hydrogel to the substrate surface, involve the formation of strong  
30 urea or urethane(carbamate) bonds. These bonds provide mechanical integrity to the microdroplet array and significantly increase the half-life of the biochip, as compared with prior art polyacrylamide-based biochips.

In certain preferred embodiments described  
35 hereinafter, the hydrogel droplets, once polymerized on

the substrate, are between about 20  $\mu\text{m}$  and 100  $\mu\text{m}$  thick; preferably, they are at least about 30  $\mu\text{m}$  thick and more preferably between about 30  $\mu\text{m}$  and about 50  $\mu\text{m}$  thick.

The overall larger size of the gel droplets (or cells)

- 5 permits a significant increase in the quantity of biomolecular probe immobilized therein, thereby increasing the sensitivity of the biochip and facilitating its use.

- In alternative embodiments contemplated herein,
- 10 water-soluble biomolecules, such as DNA or other oligonucleotides, instead of the organic-soluble biomolecules previously described, are bound to the hydrogel. By water-soluble biomolecules are meant
- 15 molecules such as DNA and RNA that are naturally water-soluble, as well as others which have been modified to be water-soluble. In these embodiments, it is not feasible to first derivatize the hydrogel prepolymer and then initiate polymerization. However, the polyurethane-based
- 20 hydrogels may advantageously be derivatized and polymerized in a single reaction while the reaction is adequately controlled to provide a derivatized hydrogel having a predictable quantity of water-soluble
- 25 biomolecular probe attached thereto. In particular, such a hydrogel prepolymer is first dissolved in an organic solvent. The DNA or other water-soluble biomolecule, in aqueous buffer solution, is then added to such prepolymer in a quantity and under appropriate conditions so that the hydrogel both becomes derivatized with the
- 30 biomolecular probe and polymerizes. As the hydrogel is polymerizing and before the polymerization is complete, it is microspotted onto a suitable substrate, as previously described.

- Alternatively, water-soluble biomolecules such as DNA, RNA and many proteins, can be chemically modified to
- 35 render them soluble in aprotic organic solvents, allowing

their derivatization to the isocyanate-functional hydrogel prior to polymerization. Modifications may include covalent modifications, such as conjugation with lipids and reversible blocking of ionic groups, as well as non-covalent modifications, such as the use of ion-pairing agents. Likewise, PNAs can be chemically modified to be satisfactorily water-soluble.

#### Optimization of Hydrogel Formulation

The swelling capacity, the polymerization time, and the transparency and strength (i.e., stability) of the ultimate polymer are important characteristics when evaluating a hydrogel for use in a biochip. Optimization of these characteristics provides an optimum hydrogel.

Swelling capacity is an important characteristic of a hydrogel because it reflects the water content. Generally, the higher the water content of the polymerized gel, the faster the diffusion of molecules in and out of the gel. For a biochip, the faster the diffusion of molecules, for example DNA samples, the more efficient the hybridization reactions. The mathematics of molecular diffusion in simple swelling polymers is based on the following semi-empirical equation (Davis B.K. Proc. Natl. Acad. Sci. USA 21:3120, 1974):

$$D_p = D_0 \exp [-0.05 + (10^{-6} M) P]$$

wherein,

$D_p$  = Diffusion coefficient of the specified molecules in the polymer solution

$D_0$  = Diffusion coefficient of the specified molecules in pure water

$M$  = Molecular weight of the specified molecules

$P$  = Percent polymer content

Thus, from this equation it can be seen that the higher the water content, the faster the diffusion of molecules

in and out of the polymer. The density and porosity of a hydrogel polymer can be controlled by a variety of parameters including reactant concentrations, the density of reactive groups, and overall reaction kinetics.

5 Optimizing the time required for polymerization of the hydrogel is particularly important in the processing of a biochip. Ideally, the time required for polymerization should be long enough to permit dispensing of the polymerizing gel onto the surface of the glass  
10 substrate with a conventional microspotting machine before polymerization is complete, but short enough that, once dispensed, the hydrogel fully polymerizes shortly thereafter. Based on these requirements, it has been determined that a hydrogel with a polymerization time of  
15 about thirty minutes and with a swelling capacity of about 96% to 97% at equilibrium exhibits good performance. Various experiments were conducted in order to determine the most appropriate formulations and reaction conditions to provide an optimum hydrogel  
20 formulation. These experiments included the evaluation of prepolymer-solvent ratios, solvent types and buffer conditions with respect to both gel transparency and polymerization rate. The results are outlined in the following sections.

25 1. Evaluation of Prepolymers and Polymer-Water Ratios

In initial experiments, it was found that hydrogel prepolymers capped with certain aromatic polyisocyanates polymerized in 2-3 minutes when treated with deionized  
30 water, i.e. too quickly for use in biochip production. In contrast, hydrogel prepolymers capped with certain aliphatic polyisocyanates took longer than 35 minutes to complete polymerization under the same conditions, causing them to be selected for further optimization. In



order to optimize the prepolymer-water ratio, varying amounts of prepolymer having an active isocyanate content of about 0.4 meq/g were dissolved in water at a pH of about 7, and the respective polymerization times were  
5 determined. As shown in Table 1, polymerization time increased as the proportion of prepolymer in the aqueous solution decreased. For example, a 5% prepolymer solution polymerized mainly on the surface, even after 48 hours. In contrast, in unbuffered DI water, the  
10 prepolymer solution having 10% prepolymer content polymerized in three hours, and the 20% solution was fully polymerized within about 35 minutes. From these initial experiments, it appeared that a prepolymer solution of at least about 5%, preferably greater than  
15 about 10% and more preferably at least about 20% should be used at this pH in the making of biochips; however, it was uncertain how the tentative conclusion might be altered by a change in pH or some other reaction condition.

20

Table 1

25

% prepolymer in aqueous solution	Polymerization time
5%	>48 hours
10%	3 hours
20%	35 minutes

## 2. Effect of pH on Polymerization

The next step toward optimization of the hydrogel formulation was to determine the effect of pH on the rate  
30 of polymerization. Table 2 summarizes results of these experiments.

Table 2

% prepolymer in aqueous solution	pH 7.2	pH 8.4
5%	90 minutes	20 minutes
10%	17 minutes	3 minutes

5  
10  
15  
In these experiments, it was found that 50 mM sodium bicarbonate aqueous buffers at pH 7.2 and 8.4 significantly accelerated the polymerization rate of the hydrogel. Thus, for example, when only a 5% prepolymer formulation was used, polymerization time decreased from >48 hours to 90 minutes at pH 7.2<sup>o</sup> to only 20 minutes at pH 8.4. Similarly, the 10% prepolymer solution was fully polymerized within 17 minutes at pH 7.2 and within 3 minutes at pH 8.4. These experiments indicated that, by adjusting the pH of the polymerization reaction solution, a lower prepolymer content, i.e. 5% or 10%, could be used in the hydrogel formulation.

20  
25  
30  
In addition to polymerization time, the swelling properties of the gel should also be considered. While polymerization may occur quickly, the gel is most useful when it has reached the desired water content of at least about 95%, preferably about 96% to 97%. Thus, the swelling characteristics of the different polymer content formulations were also analyzed, and Table 2A compares the swelling profiles of a 5% hydrogel formulation and a 10% hydrogel formulation. Water content is reported in terms of swelling ratio, and swelling ratio is calculated by dividing the total weight of the hydrogel by the weight of the prepolymer.

Table 2A  
Swelling Ratio as a Function of Time and % Prepolymer

	Hours			
Composition	1	1½	3	24
5 % prepolymer	23	25	27.5	40
10% prepolymer	12	13	15	25

From these test results, it can be seen that the hydrogel made using a 10% prepolymer solution required about one day to achieve an optimum water content of 96%, which is equal to a swelling ratio of about 25. In addition, the volume of hydrogel, when fully swollen, was about twice its volume just after polymerization began, making the structural integrity of this hydrogel difficult to maintain. In contrast, the hydrogel resulting from a 5% prepolymer solution reached 96% water content in about one hour and did so without loss in structural integrity. Thus, based upon both polymerization rate in this slightly basic solution and swelling profile, the 5% prepolymer solution was selected for still further optimization.

### 3. Optimizing Transparency of the Hydrogel

A significant disadvantage of most hydrogels made from aqueous solutions is their general opaqueness. To be most useful in a biochip, the gel should be transparent, and in particular, it should be optically transparent so that interference with certain molecular markers or labels, such as fluorescent tags, is minimized. To achieve optical transparency, the hydrogel prepolymer was first dissolved in an aprotic organic solvent, such as acetonitrile, acetone, DMF, NMP or a mixture thereof, and the resulting solution was treated with 50 mM sodium bicarbonate buffer at pH 7.2 or 8.4 to

initiate polymerization.  $\text{NaHCO}_3$  will normally buffer an aqueous solution at a pH of about 8.4; however, the addition of an acid, e.g.  $\text{HCl}$ , or a base, e.g.  $\text{NaOH}$ , can be used to slightly alter the buffered pH, e.g. to expand the range to extend from 7.0 to 9.5, although some of the buffer capacity is lost. Using the preferred 5% polymer solution, the ratio of aprotic solvent to aqueous buffer was adjusted to determine an optimum formulation with respect to polymerization time and optical transparency. Table 3 shows the results of testing of five different hydrogel formulations having various ratios of buffer to aprotic solvent, in this case acetonitrile. Each formulation was tested at a buffered pH of 8.4 or 7.2.

Table 3

15

20

25

Formulation #	Acetonitrile: Buffer	Time to polymerize
DI water		
control	0.3 g:0.65 g	3 hours
pH 8.4 buffer		
1	0.1 g: 0.85 g	45 minutes
2	0.2 g: 0.75 g	42 minutes
3	0.3 g: 0.65 g	35 minutes
4	0.4 g: 0.55 g	35 minutes
5	0.5 g: 0.45 g	32 minutes
pH 7.2 buffer		
6	0.1 g: 0.85 g	180 minutes
7	0.2 g: 0.75 g	170 minutes
8	0.3 g: 0.65 g	180 minutes
9	0.4 g: 0.55 g	180 minutes
10	0.5 g: 0.45 g	150 minutes

Each of the tested formulations at pH 8.4 and pH 7.2 polymerized within about 30-45 minutes and 150-180 minutes, respectively, regardless of the relatively large

differences in the ratio of acetonitrile to buffer. The higher the pH, the faster the polymerization. It was found that the pH of an earlier prepared sodium bicarbonate buffer increased over time due to the release of CO<sub>2</sub> gas from the buffer; thus, when sodium bicarbonate is used as a buffer system, it should be prepared freshly and used the same day. Another buffer system, 50 mM borate buffer, was tested and found to achieve complete polymerization in a time similar to that of freshly-made sodium bicarbonate buffer. A borate buffer is an aqueous solution containing sodium tetraborate and boric acid wherein the proportions can be varied to provide a pH between about 7.0 and about 9.5.

Visual transparencies of each resulting hydrogel varied with respect to the ratio of organic solvent to aqueous buffer. Transparency is believed to be affected by evolution of CO<sub>2</sub> and/or by precipitation of the prepolymer. The visual transparency appeared to improve as the ratio of organic solvent to aqueous buffer was increased, which prompted the following additional experiment to be performed.

To measure the effect of visual opacity of the hydrogel upon the optical intensity of fluorescence, the following experiment was performed using three formulations (10%, 20% and 30% aprotic solvents by weight).

Formulation I:

0.2 g Hypol PreMa G-50 was dissolved in 0.22 g acetonitrile and 0.22 g of NMP. The resulting solution is reacted with a DNA solution in 3.8 ml of 50 mM borate buffer pH 8.0 (oligonucleotide concentration, G11, i.e. 5'-CTAAACCTCCAA-3' of 0.75 mg/ml of the buffer). The formulation contained about 10 weight % organic solvents.

Formulation II:

0.2 g Hypol PreMa G-50 was dissolved in 0.44 g acetonitrile and 0.44 g of NMP. The resulting solution

is reacted with DNA solution in 3.36 ml of 50 mM borate buffer at pH 8.0 (oligonucleotide concentration, G11, of 0.84 mg/ml of the buffer). The formulation II contained about 20 weight % organic solvents.

5 Formulation III:

0.2 g Hypol PreMa G-50 was dissolved in 0.67 g of acetonitrile and 0.67 g of NMP. The resulting solution is reacted with DNA solution in 2.89 ml of 50 mM borate buffer at pH 8.0 (oligonucleotide concentration, G11, of 10 1 mg/ml of the buffer). The formulation III contained about 30 weight % organic solvents.

Formulations I, II and III were microspotted onto glass slides using a conventional microspotting machine, as explained in more detail hereinafter in Example III. 15 After 1 hour curing in a controlled humidifier chamber at a relative humidity of about 95%, the resulting slides were washed with washing buffer and hybridized with fluorescently-labeled target sample for 20 minutes. The intensity of each formulation was measured with a CCD 20 camera. The results are described in Table 3A from which it is clear that the opacity is dependent upon the relative percentage of organic solvent and affects the fluorescence intensity.

25 Table 3A. Effect of Visual Opacity on the Intensity of Fluorescence

(organic solvents)	Visual opacity	Fluorescence intensity
Formulation I (10%)	Opaque	2044 (+217)
Formulation II (20%)	Moderately opaque	8223 (+779)
Formulation III (30%)	Transparent	10209 (+632)

30 As a result of the above testing, it was determined that hydrogel formulations employing at least about 20% and preferably at least about 30% organic solvent by weight of the total solution which is polymerizing and preferably at least about 15% acetonitrile are preferred 35 when optical clarity is desired in the ultimate biochip.

#### 4. Comparison of Aprotic Organic Solvents

The next step in optimization of the hydrogel for the biochips of the present invention, was a comparison of aprotic solvents. Experiments similar to those described in Section II were performed using various aprotic solvents, namely DMF, NMP and acetone. While it was assumed these alternative organic solvents would function similarly to acetonitrile, such was not the case. Results of polymerization studies of hydrogel formulations having 30% DMF, NMP or acetone are summarized in Table 4.

Table 4

Solvent	pH of buffer	Polymerization time
DMF	unbuffered DI water	Did not polymerize
	8.4	Did not polymerize
	9.15	20 minutes
	9.5	8 minutes
	10	Did not polymerize
NMP	unbuffered DI water	Did not polymerize
	8.4	Did not polymerize
	9.15	Did not polymerize
	9.5	Did not polymerize
	10	Did not polymerize
Acetone	unbuffered DI water	Did not polymerize
	8.4	30 minutes

From these experiments, it was discovered that formulations in DMF required a higher pH to polymerize while, under the same conditions, formulations in NMP did not polymerize at all. Formulations in acetone were most similar to acetonitrile formulations in that they required only slightly basic pH to polymerize.

Significantly, the polymerized hydrogels resulting from DMF or acetone formulations were inferior in structural integrity to the polymerized hydrogels produced from acetonitrile formulations. Thus, from these optimization  
5 studies, acetonitrile was selected as the preferred aprotic solvent for use in the further development of the optimized biochip; later experiments showed that other aprotic organic solvents such as tetrahydrofuran and dioxane were found to exhibit a similar propensity for  
10 achieving complete polymerization as that of acetonitrile and acetone.

Based upon all of the previously described tests, it is generally believed that the pH of the polymerizing solution should be between about 7.0 and about 9.5,  
15 preferably between about 7.2 and about 8.6, more preferably between about 7.4 and about 8.4 and most preferably between about 7.6 and about 8.2.

#### 5. Evaluation of Humidity Effects During Polymerization

20 During or after microspotting, the deposited microdroplets should avoid extensive dehydration or immediate loss of organic solvent. Such loss may result in over-crosslinking during polymerization on the substrate and resultant low diffusability of large  
25 molecular weight target samples. This potential problem is reduced by placing the polymerizing biochip into a controlled humidity chamber immediately after microspotting. A controlled humidifier was constructed and maintained at approximately 97 to 98% humidity as  
30 determined with a digital sling psychrometer (Model THWD-1, Amprobe Instrument). After microspotting, the biochip was immediately placed within the humidifier chamber. Alternatively, the controlled humidity chamber could be extended to the microspotting process environment.



Table 5 shows the effect of humidity during polymerization on the ultimate intensity of fluorescence of bound biomolecules.

Table 5

## 5      The Effect of Humidity During Polymerization

Condition	Fluorescence Intensity
Dry	762 ( $\pm 207$ )
Humidity 97-98%	7161 ( $\pm 2454$ )

The test shows that there is a nearly 10-fold decrease in  
10 intensity when polymerization is carried out under dry  
conditions. This difference is attributed to overcross-  
linking of the polymerized microdroplet when humidified  
conditions are not employed and consequently low  
diffusability of target samples. It is thus preferred to  
15 expose the microspotted biochip to at least about 90%  
relative humidity during polymerization.

6.    Temperature Control of Hydrogel Polymerization

After the prepolymer mixes with aqueous DNA buffer  
solution, polymerization begins to take place, resulting  
20 in a slow increase in the viscosity of the mixture while  
it is being applied to the biochip substrate. Although  
continuous mixing and dispensing processes can be used,  
it has been found that simple control of reaction  
kinetics will allow a batch process to be used for  
25 preparing and microspotting the polymerizing hydrogel.  
At room temperature, a significant viscosity change was  
observed during a five to ten-minute time period, which  
may affect the size and height of microdroplets during  
dispensing. This potential problem was essentially  
30 eliminated by microspotting in a cold box at 7°C.  
Lowering the temperature slowed down the polymerization  
process resulting in more consistent viscosity.

0.2 g of isocyanate-functional hydrogel Hypol PreMa G-50 was dissolved in 0.67 g acetonitrile and 0.67 g N-methyl-2-pyrrolidinone, and the resulting mixture was polymerized by adding 2.89 g of 50 mM borate buffer at pH 8.0. The polymerization was performed at room temperature (~24°C) and at 7°C. The time to completely polymerize was observed and is presented in Table 6.

Table 6  
Polymerization Time at Different Temperatures

10	Temperature	Polymerization Time
	Room temperature	50 minutes
	7°C	2 hours

This testing shows that complete polymerization can be delayed by subjecting the microdroplets to lower temperature, and preferably a temperature of not greater than about 10°C is used.

#### 7. Effect of Thickness on Sensitivity

A solution was prepared by dissolving 0.2 g of Hypol PreMa G-50 in 0.67 acetonitrile and 0.67 g N-methyl-2-pyrrolidinone. The resulting solution was reacted with a DNA solution in 2.89 ml of 50 mM borate buffer at pH 8.0; the DNA was in the form of the oligonucleotide G11, i.e. 5'-CTAAACCTCCAA-3', at a concentration of 1 mg/mL of the buffer. The overall formulation contained about 30% organic solvents by total weight.

The polymerizing solution of hydrogel was microspotted onto glass slides using a conventional microspotting machine. After curing in the controlled humidifier chamber for 1 hour, resulting slides were washed with washing buffer and then hybridized with a fluorescently-labeled target sample for 20 minutes. The

intensity of each formulation was measured with a CCD camera.

Three different heights, 21 $\mu$ m, 32 $\mu$ m and 52 $\mu$ m, of hydrogel microdroplets were produced using the same hydrogel formulation by appropriately adjusting the microspotting mechanism. The heights of microdroplets were measured with a thickness measurement instrument, KLA-Tencor P11. The hydrogel cells were hybridized using standard procedure, and the resulting fluorescence intensities were detected optically and measured. The results are summarized in Table 7.

Table 7  
Effect of Hydrogel Thickness on Fluorescence

Thickness ( $\mu$ m)	Fluorescence Intensity
21	2728 ( $\pm$ 480)
32	4404 ( $\pm$ 640)
52	5616 ( $\pm$ 432)

The results of the tests show that the thicker the hydrogel cell, the greater the sensitivity.

#### 20 Derivatization of the Hydrogel with a Biomolecular Probe

##### Derivatization with a Peptide Nucleic Acid Probe

##### A. Optimization of Reaction Conditions

Having optimized the hydrogel formulation and its polymerization as indicated, attention was turned to derivatizing the gel with an appropriate biomolecular probe to create a biochip with PNAs, which are considered to be more stable. PNA probes were thus generated for use in derivatizing a preferred hydrogel formulation.

PNAs were first dissolved in acetonitrile, a preferred aprotic solvent, for preparing the hydrogel, but it was discovered that PNAs are only minimally soluble in acetonitrile. A more polar solvent, i.e. NMP,

was selected for trial, even though hydrogel formulations using NMP did not polymerize under various of the conditions tested, to see if a solution containing both NMP and acetonitrile might be capable of optimization to provide both adequate solubility for PNAs and favorable polymerization for the hydrogel. Numerous experiments with differing acetonitrile/NMP ratios, determined that an optimum formulation should have a solvent ratio in the range of 3:1 to 1:1 acetonitrile to NMP when those two aprotic solvents were used. An aqueous solution of 50 mM sodium bicarbonate was then used to buffer the polymer/PNA solution at about pH 8.0, creating a formulation which completely polymerized in 20-30 minutes. FIG. 1 illustrates, schematically, the reaction of the hydrogel prepolymer with an organic-soluble biomolecule, such as PNA, and the polymerization of the derivatized prepolymer.

#### B. Effect of PNA Derivatization on Polymerization of the Hydrogel

The next step in providing a PNA biochip, in accordance with this first preferred embodiment, was to evaluate the extent to which the degree of derivatization with PNA affects polymerization of the hydrogel. It was generally considered that the hydrogel formulation in acetonitrile should have an active isocyanate content of at least about 0.1 meq/g and not greater than about 1 meq/g, and that about 0.2-0.8 meq/g would likely be preferred. Using prepolymers within this range, experiments were performed wherein different percentages of the active isocyanates were derivatized with a test compound, benzylamine, for five minutes; the hydrogel used an active isocyanate concentration of about 0.4 meq/g. The derivatized hydrogels were then polymerized by treating with 50 mM sodium bicarbonate buffer at pH 8.0. Derivatization of 10% of the active isocyanates with benzylamine resulted in a polymerized hydrogel

having characteristics similar to those of the underivatized, polymerized hydrogel. In contrast, when at least about 20% of the active isocyanates were derivatized with benzylamine, the resulting formulation  
5 did not polymerize, indicating that an insufficient quantity of active isocyanates were thereafter available for polymerization. Thus, it is felt that derivatization of about 10% of the active isocyanates of the gel is preferred and that not more than about 15% of the active  
10 isocyanates should be so reacted. When 10% of the active isocyanates are derivatized with PNA, it is estimated that about 1 to 2 pmol of PNAs will be bound within a droplet of hydrogel of about 1 nanoliter. Control of this parameter is effected by the amount of PNA or other  
15 biomolecule added to the prepolymer solution.

#### Derivatization with a DNA Probe

In an alternative embodiment, the hydrogel of the present invention is derivatized with a DNA (or similar oligonucleotide) probe. Unlike PNA however, DNA is not  
20 soluble in organic solvent. Therefore, where DNA is used as the biomolecular probe, the derivatization and polymerization steps are not separated but are carried out as a part of a single step. Additionally unlike PNA, DNA does not have an active amine (or other) group  
25 available for binding to the active isocyanates of the polymer; thus, DNA is preferably modified, prior to reaction with the prepolymer, to add an appropriate active group, such as by reaction with a diamine or other reagent that would provide an active primary amine, as by  
30 using standard amidite chemistry.

As with the previously described embodiment wherein the hydrogel was derivatized with PNA, the organic solvent and aqueous buffer solution amounts and reaction conditions are selected to optimize the hydrogel  
35 polymerization with respect to polymerization time, gel

transparency and related characteristics. A polymer content of about 4-10% and an organic solvent content of at least about 20% (more preferably at least about 30%) are found to be preferred. It is similarly felt no more than about 20% should be derivatized, preferably not more than about 15%, and more preferably, about 10% or less, of the active isocyanates of the hydrogel, by linking with the DNA probe. Thus, for example, as exemplified below, about 2.5% of the active isocyanates of the hydrogel may be derivatized with the DNA probe, with polymerization preferably being accomplished using a basic buffered aqueous solution, at about pH 7.2 to about 8.6, and more preferably about 7.6 to about 8.2.

To prepare this derivatized hydrogel, the prepolymer is first dissolved in an organic solvent, for example, acetonitrile, and the derivatized DNA (or other water-soluble oligonucleotide) probe is prepared in aqueous buffer. The DNA solution is then added to the prepolymer solution with thorough mixing, resulting in both derivatization of the gel and initiation of polymerization. This general method of dissolving a polyurethane-based hydrogel in organic solvent and adding thereto a water-soluble biomolecular probe in aqueous solution is applicable to numerous water-soluble probes in addition to oligonucleotides. FIG. 2 illustrates, schematically, the simultaneous reaction of a water-soluble biomolecule, such as DNA, with the hydrogel prepolymer and polymerization of the hydrogel.

#### Binding of Hydrogel to the Surface of the Substrate

The final step is that of dispensing the derivatized hydrogel prepolymer formulation onto a suitable substrate under appropriate conditions to permit binding of the hydrogel to the substrate surface. When the substrate selected is transparent, in order to reduce interference with signal detection when the biochip is scanned by

transmitted light, a preferred substrate is glass, the surface of which is first derivatized with amine, in order to provide active sites for covalent binding of the polymerizing hydrogel. Just prior to dispensing the

5 derivatized hydrogel onto the derivatized glass substrate, polymerization is initiated either by addition of aqueous buffer to the derivatized prepolymer (in the case of derivatization with an organic-solvent-soluble biomolecule) or by addition of aqueous buffer which

10 includes a water-soluble biomolecule. The polymerizing hydrogel is then microspotted onto the glass substrate to form an array of microdroplets most preferably having a thickness of about 30 to 50  $\mu\text{m}$ .

A 50 mM sodium bicarbonate aqueous buffer solution,

15 pH from about 7.2 to about 8.4, may be used to initiate polymerization of the hydrogel, and the hydrogel formulation binds tightly to the surface of such derivatized glass within about 1 to 10 minutes from the initiation of polymerization. When PNA or another

20 organic-soluble probe is used, the derivatized prepolymer need not be used immediately following derivatization, but it may be stored under proper conditions prior to initiation of polymerization by addition of aqueous buffer. In contrast, when the prepolymer is derivatized

25 with a water-soluble probe, polymerization and derivatization begin upon addition of the probe to the prepolymer solution so the polymerizing hydrogel should be dispensed shortly thereafter.

Advantageously, these isocyanate-functional

30 hydrogels polymerize quickly into very stable microdroplets, and such are preferably dispensed onto a substrate to form microdroplets of at least about 20  $\mu\text{m}$  in height, more preferably at least about 30  $\mu\text{m}$  in height, and most preferably about 30  $\mu\text{m}$  to about 50  $\mu\text{m}$  in

35 height. Consistency between hydrogel droplets is obtained by dispensing the polymerizing gel using a microspotting

or similar automated machine, as providing such thick gel droplets, or gel cells, can dramatically increase the sensitivity of the biochip.

In order to prevent overcrosslinking of the polymerizing gel, which can result in an increase in the polymer concentration as water is evaporated from the gel and which may also cause wrinkling in the surface of the gel droplet, the biochip is preferably cured in a humid environment, such as a humidity chamber, and thereafter washed with deionized water.

The viscosity and sheathing properties of the polymerizing hydrogel against the glass substrate may be important to provide a reasonable and consistent shape of droplets throughout the array. In an ideal situation, the viscosity of the polymerizing hydrogel increases slowly and in a linear fashion. Unfortunately, however, the viscosity of hydrogels tend to increase exponentially during polymerization. To facilitate a more linear expansion of the hydrogel during polymerization, its viscosity may be modified using thickening agents, such as polyethylene glycol of various molecular weights. The viscosity of the hydrogel during polymerization may be measured, for example with a viscometer, following addition of the thickening agent. From experimentation, it was determined that addition of polyethylene glycol having a molecular weight of about 1,000 or higher, e.g. as high as the molecular weight of the prepolymer, could significantly improve the formulation's viscosity and sheathing properties. Thus, in certain preferred embodiments, polyethylene glycol or a similar thickening agent is added to the hydrogel during polymerization thereof to control the rate of polymerization.

#### Example I: Preparation of a PNA Biochip and Test

A solution of 1 mg PNA (0.3  $\mu$ m) having the sequence NH<sub>2</sub>-CATTGCTCAAAC-CO<sub>2</sub>H was prepared in 0.1 g of NMP. Next,



a solution of 0.05 g of Hypol PreMa G-50, a polyurethane prepolymer having an active isocyanate content of about 0.4 meq/g, was prepared in 0.2 g acetonitrile and added to the PNA-NMP solution. The resulting solution was  
5 treated with 0.65 g of a 50 mM NaHCO<sub>3</sub> solution at about pH 8.0. After thorough mixing, two droplets of the resulting solution were manually spotted on a silanated glass slide using a capillary microtube. The droplets polymerized on the surface of the glass in about 15  
10 minutes. As a negative control, two hydrogel droplets containing no PNA were spotted next to the PNA-containing hydrogel droplets.

The glass slide, having the hydrogel droplets thereon, was submersed into washing buffer (5 mM sodium  
15 phosphate buffer with 0.05% SDS at pH 7.0) for 30 minutes to remove organic solvents and block the remaining active sites to prevent non-specific binding of test DNA. Next, the slide was treated with 1 mg of a complementary fluorescence-labeled DNA, 3'-TAGTAACGAGTTTGCC-5'-  
20 Fluorescein, in hybridization buffer containing 600 µL of 10 mM sodium phosphate buffer with 0.1% SDS at pH 7.0 at room temperature, for 1 hour. Non-specifically bound DNA was removed by washing for two hours in washing buffer. The resulting slide was observed with a hand-held  
25 fluorescence detector (Model UVGL-25, UVP). The test DNA diffused into the hydrogel microdroplets and hybridized to the complementary PNA capture probe sequence, providing a strong fluorescent signal, while the test DNA had been washed away from the negative control droplets  
30 which gave no signal. The test demonstrates the usefulness of such hydrogel biochips.

**Example II: Preparation of a DNA Biochip and Test**

A solution of 0.025 g of Hypol PreMa G-50 was prepared in 0.15 g acetonitrile. Next, a solution of 1  
35 mg DNA (0.3 µm), having hexaneamine at its 5' end and

having the sequence  $\text{NH}_2(\text{CH}_2)_6\text{-CATTGCTCAAAC-3'}$ , in 0.32 g of a 50 mM  $\text{NaHCO}_3$  aqueous buffer at pH 8.0 was prepared. The DNA solution was added to the prepolymer solution and thoroughly mixed. Droplets of the resulting solution were manually spotted on a silanated glass slide using a capillary microtube. As a negative control, hydrogel droplets containing no DNA were spotted next to the DNA-containing hydrogel droplets.

The glass slide, having the hydrogel droplets thereon, was submersed into washing buffer (10 mM sodium phosphate buffer with 0.5 M NaCl and 0.1% SDS at pH 7.0) for 30 minutes to remove organic solvents and block the remaining active sites to prevent non-specific binding of test DNA. Next, the slide was treated with 1 mg of a complementary fluorescence-labeled DNA, 3'-TAGTAACGAGTTTGCC-5'-Fluorescein, in 600  $\mu\text{L}$  hybridization buffer (10 mM sodium phosphate buffer with 0.5 M NaCl and 0.1% SDS at pH 7.0) at room temperature, for 1 hour. Non-specifically bound DNA was removed by washing for two hours in washing buffer. The slide was observed with a hand-held fluorescence detector (Model UVGL-25, UVP). The complementary, test DNA diffused into the hydrogel microdroplet and hybridized to the gel-bound DNA probe sequence resulting in a strong fluorescent signal, but it was washed away from the negative control droplet, demonstrating the reliability and usefulness of the present hydrogel biochips in DNA hybridization assays.

**Example III. Preparation of an Array DNA Biochip and Test in Human  $\beta$ -globin Gene Sequence Detection**

A DNA biochip was prepared as follows:

1. The following two reactant solutions were prepared:

Solution A = 0.1 g Hypol Pre-Ma G-50 in 0.33 g acetonitrile and 0.33 g NMP (Weight ratio of 4.5:15:15)

Solution B = 1 mg of oligonucleotide in 1 ml of 50 mM borate buffer at pH 8.0

2. Solution A (34.5 parts) was mixed with Solution B (65.5 parts), and the resultant solution  
5 microspotted onto a glass slide. Microspotting was performed with an open configuration pin, CT MicroPipets DP-120  $\mu\text{m}$ , supplied by Conception Technologies.

3. The microspotted slides were placed into a controlled humidifier chamber for one hour and then  
10 washed with a washing buffer for 10 minutes, completing the preparation of the biochips.

Testing of such a biochip is performed by hybridization with a target sample carrying a fluorescent tag or the like at different concentrations in a  
15 hybridization buffer system for 20 minutes to 2 hours, proportional to the molecular weight of the target. Any non-specifically bound target is washed away with the hybridization buffer, and the biochip is then scanned to detect the bound target by optical fluorescence.

20 To validate the performance of these biochips which carry DNA probes, the following twenty 12-mer oligonucleotides, derivatized with primary amine at the respective 5' end using standard amidite chemistry, were immobilized on separate hydrogel cells as a part of a  
25 biochip made in this manner:

	G1	5'-CCTAAGTTCATC-3'
	G2	5'-TATCTCTTATAG-3'
	G3	5'-CTATCGTACTGA-3'
	G4	5'-TTCCTTCACGAG-3'
5	G5	5'-ATTATTCCACGG-3'
	G6	5'-ATCTCCGAACTA-3'
	G7	5'-CCTTATTATGCA-3'
	G8	5'-ACGCTTCCTCAG-3'
	G9	5'-GACTTCCATCGG-3'
10	G10	5'-CGTACCTTGTA-3'
	G11	5'-CTAAACCTCCAA-3'
	G12	5'-CTAGCTATCTGG-3'
	G13	5'-TAATTCCATTGC-3'
	G14	5'-ATTCCGATCCAG-3'
15	G15	5'-TTAGTTATTCTGA-3'
	G16	5'-AAGTTCATCTCC-3'
	G17	5'-TTCATCTCCGAA-3'
	G18	5'-CCGAACTAAACC-3'
	G19	5'-AACTAAACCTCC-3'
20	G20	5'-CTAAACGTCCAA-3'
	G21	Blank hydrogel

A target 30-mer DNA sample from the sequence of the human  $\beta$ -globin gene was synthesized and labeled with a tagging molecule, i.e. fluorescein, at its 5' end using standard amidite chemistry. The sequence of this target sample is the following:

5'-(Fluorescein)-TTGGAGGTTTAGTTCGGAGATGAACTTAGG-3'

The sequences of G1, G6, G11, G16, G17, G18 and G19 are fully complementary to different regions of the target sample. The sequence of G20 has an internal one-base pair mismatch from that of G11. The results of the testing are set forth in Table 8 which follows:

Table 8  
The Intensity of Fluorescence Depending on Sequences

	Oligonucleotide	G1	G6	G11	G16	G17	G18	G19	G20
	Intensity	1528	2713	5630	650	841	2098	6066	2181
5	Standard deviation	77	151	238	164	127	354	638	225

As seen in Table 8, the hybridization discrimination of perfect match (G11) and one-base pair mismatch (G20) was excellent (Fluorescence intensity of 5630 vs 2181). The non-related oligonucleotides of G2, G3, G4, G5, G7,  
10 G8, G9, G10, G12, G13, G14 and G15, as well as the blank hydrogel cell, demonstrated intensity just above background showing minimum non-specific binding to the hydrogel.

The disclosures of all U.S. patents set forth herein  
15 are expressly incorporated herein by reference.

The methods for making isocyanate-functional biochips, the biochip systems and the hybridization assays described herein have been described in terms of particular embodiments which encompass the best modes presently contemplated. However, it will be appreciated by those of skill in the art that certain parameters, such as the particular aprotic solvent selected, the polymerization time desired and/or used, and the biomolecular probe selected for binding to the hydrogel may be altered without departing from the nature of the invention as described herein. Thus, the scope of the invention should be determined not with reference to the above description alone but with reference to the appended claims along with their full scope of equivalents.

## CLAIMS:

1. A method of preparing a hydrogel biochip having a biomolecule immobilized thereon, the method comprising the steps of:
  - (a) providing an organic solvent solution of isocyanate-functional hydrogel prepolymer;
  - (b) providing a solution of a biomolecule;
  - (c) covalently binding the biomolecule to the hydrogel prepolymer; and
  - (d) initiating polymerization of the hydrogel prepolymer.
2. The method according to claim 1 wherein the substrate has active amines on its top surface which covalently bind the hydrogel to the substrate.
3. The method according to claim 1 wherein the hydrogel prepolymer is provided as a solution of less than about 10% hydrogel prepolymer having active isocyanates of at least about 0.1 meq/g.
4. The method according to claim 3 wherein the solution contains no more than about 5% of a prepolymer which comprises polyethylene oxide or a copolymer of polyethylene oxide and polypropylene oxide capped with diisocyanates, which prepolymer may be optionally lightly cross-linked, and wherein the prepolymer contains less than about 1 meq/g active isocyanates.
5. The method according to claim 1 wherein the hydrogel prepolymer is provided in an aprotic organic solvent selected from the group consisting of N-methyl-2-pyrrolidinone (NMP), dimethylformamide (DMF), acetonitrile, acetone, tetrahydrofuran, dioxane and mixtures thereof.

6. The method according to claim 5 wherein the hydrogel prepolymer is dissolved in acetonitrile.

7. The method according to claim 1 wherein the steps of covalently binding the biomolecule to the hydrogel prepolymer and initiating polymerization of the hydrogel prepolymer are performed simultaneously.

8. The method of claim 7 wherein the biomolecule comprises DNA, RNA or PNA in aqueous solution.

9. The method according to claim 8 wherein the aqueous solution is buffered at a pH from about 7.0 to about 9.5.

10. The method according to claim 1 wherein the reaction conditions are used to control carbon dioxide evolution and to avoid precipitation of isocyanate-functional prepolymer to minimize opacity of the resulting hydrogel, and wherein polymerization is carried out under at least about 90% relative humidity.

11. A biochip made according to the method of claim 1 wherein the biomolecular probe comprises DNA, RNA or PNA, and wherein no more than about 20% of the reactive isocyanates in the prepolymer are used to immobilize the biomolecular probes.

12. The biochip of claim 11 wherein about 10% or less of the reactive isocyanates in the prepolymer are used to immobilize the biomolecular probes and other of the reactive isocyanates are used to covalently bind to the substrate and to form a cross-linked hydrogel.

13. A method of preparing a biochip having an organic solvent soluble biomolecule covalently bound thereto, the method comprising:

- (a) providing a solid substrate having a top surface;
- (b) providing a solution comprising the biomolecule in an aprotic organic solvent;
- (c) providing an isocyanate-functional hydrogel prepolymer in an aprotic organic solvent;
- (d) derivatizing the prepolymer with the biomolecule from step (b);
- (e) initiating polymerization of the derivatized hydrogel prepolymer of step (d) by adding a buffered aqueous solution thereto; and
- (f) microspotting the polymerizing hydrogel solution of step (e) onto the top surface of the substrate of step (a), resulting in attachment of the hydrogel and immobilized biomolecule to the substrate.

14. The method according to claim 13 wherein the biomolecule is a peptide nucleic acid which is dissolved in an aprotic organic solvent selected from the group consisting of N-methyl-2-pyrrolidinone (NMP), dimethylformamide (DMF), acetone and mixtures thereof.

15. The method according to claim 14 wherein the peptide nucleic acid is dissolved in NMP.

16. The method according to claim 13 wherein step (d) comprises reacting a solution of peptide nucleic acid in NMP with a solution of hydrogel prepolymer in acetonitrile.

17. The method according to claim 16 wherein the ratio of acetonitrile to NMP in the derivatization step is between about 3:1 and about 1:1.



18. The method according to claim 13 wherein the step of providing a hydrogel prepolymer in an aprotic organic solvent comprises providing a polyurethane-based prepolymer in acetonitrile and wherein the polymerizing solution of step (e) contains at least about 15% acetonitrile.

19. The method according to claim 13 wherein the substrate is derivatized with an amine, wherein step (c) provides a polyurethane-based hydrogel prepolymer in an organic solvent which comprises acetonitrile and wherein the polymerizing solution of step (e) contains at least about 20% organic solvent.

20. The method according to claim 13 wherein the active isocyanate content of the hydrogel prepolymer is between about 0.2 and 0.8 meq/g.

21. The method according to claim 14 wherein step (d) comprises derivatizing about 15% or less of the active isocyanates of the prepolymer with the peptide nucleic acid and wherein the ratio of organic solvent to water in step (c) is greater than 0.25 to 1.

22. The method according to claim 21 wherein no more than about 10% of the active isocyanates of the hydrogel are derivatized with peptide nucleic acid and wherein step (e) comprises adding an aqueous solution to the derivatized prepolymer solution to provide a resultant solution which is buffered at a pH from about 7.4 to about 8.4.

23. The method according to claim 21 wherein step (a) comprises providing a solution of polyurethane-based hydrogel prepolymer in acetonitrile, wherein the hydrogel prepolymer has an active isocyanate content of not

greater than about 0.8 meq/g; wherein step (b) comprises providing a solution of peptide nucleic acid in NMP, wherein the concentration of peptide nucleic acid is equal to at least about 10% of the concentration of active isocyanates in the hydrogel prepolymer solution and the volume of NMP is at most about one half the volume of the acetonitrile; and wherein in step (c) the hydrogel prepolymer solution and the peptide nucleic acid solution react such that not more than about 20% of the active isocyanates of the hydrogel prepolymer become covalently bound to the biomolecule.

24. A biochip comprising:

- (a) a solid substrate having a top surface;
- (b) a plurality of hydrogel cells covalently bound to the top surface of the substrate, wherein each hydrogel cell is formed of an isocyanate-functional polymer; and
- (c) a biomolecular probe covalently bound to and within at least one of the hydrogel cells.

25. The biochip of claim 24 wherein each hydrogel cell comprises a polymer with urethane and urea linkages and is at least about 20  $\mu\text{m}$  thick.

26. The biochip of claim 24 wherein the hydrogel polymer comprises polyethylene glycol, polypropylene glycol, or a copolymer thereof.

27. The biochip of claim 24 wherein the substrate is transparent and has reactive molecules on its top surface which covalently bind the hydrogel to the substrate and wherein each hydrogel cell is between about 20  $\mu\text{m}$  and about 100  $\mu\text{m}$  thick.

28. A biochip comprising:  
    (a) a solid substrate having a top surface;  
    (b) a plurality of hydrogel cells covalently bound to the top surface of the substrate, wherein the hydrogel comprises polyethylene glycol, polypropylene glycol, or a copolymer thereof; and  
    (c) different biomolecular probes which are covalently bonded to and within different hydrogel cells.

29. A biochip according to claim 28 wherein each hydrogel cell is at least 20  $\mu\text{m}$  thick and wherein the biomolecular probe comprises DNA, RNA or PNA.

30. A biochip according to claim 28 wherein the hydrogel cell is between about 30  $\mu\text{m}$  and 50  $\mu\text{m}$  thick.

31. A biochip comprising:  
    (a) a solid substrate having a top surface;  
    (b) a plurality of hydrogel cells covalently bound to the top surface of the substrate wherein the hydrogel comprises a polymer with urethane-urea linkages; and  
    (c) different biomolecular probes which are covalently bound to and within different hydrogel cells.

32. The biochip of claim 31 wherein the hydrogel cell is at least 20  $\mu\text{m}$  thick and wherein the biomolecular probe comprises DNA, RNA, or PNA.

33. A hybridization assay comprising:  
    (a) providing a biochip which comprises a substrate having at least two hydrogel cells bound thereto, each cell having a thickness of at least about 20  $\mu\text{m}$  and comprising a major portion of a polymer selected from the group consisting of polyethylene glycol, polypropylene glycol and copolymers thereof,

wherein at least one hydrogel cell further includes a biomolecular probe covalently bound thereto;

(b) contacting the biochip with an analyte solution, containing a target biomolecule, under hybridization conditions;

(c) washing the hydrogel biochip under conditions that remove non-specifically bound and unbound target biomolecule; and

(d) detecting the bound target biomolecule.

34. A hybridization assay according to claim 33 wherein the step of detecting the bound target biomolecule further comprises contacting the biochip with a tagging molecule, wherein the tagging molecule selectively binds to the target molecule, and detecting the tagging molecule.

35. A hybridization assay according to claim 33 wherein the analyte solution contains a target biomolecule having a fluorescent marker bound thereto and wherein the step of detecting the bound target biomolecule comprises detecting fluorescence from the fluorescent marker.

36. A hybridization assay comprising:

(a) providing an organic solvent solution of a hydrogel prepolymer having an active isocyanate content of not greater than about 1 meq/g;

(b) providing a solution of a biomolecule;

(c) covalently binding the biomolecule to at least a portion of the hydrogel prepolymer;

(d) initiating polymerization of the hydrogel prepolymer;

(e) dispensing the polymerizing hydrogel prepolymer onto a solid substrate such that said solution

is covalently bound to the substrate at a thickness of at least about 20  $\mu\text{m}$ , thereby resulting in a biochip;

(f) washing the biochip from step (e) with washing buffer such that remaining active sites within the hydrogel are blocked;

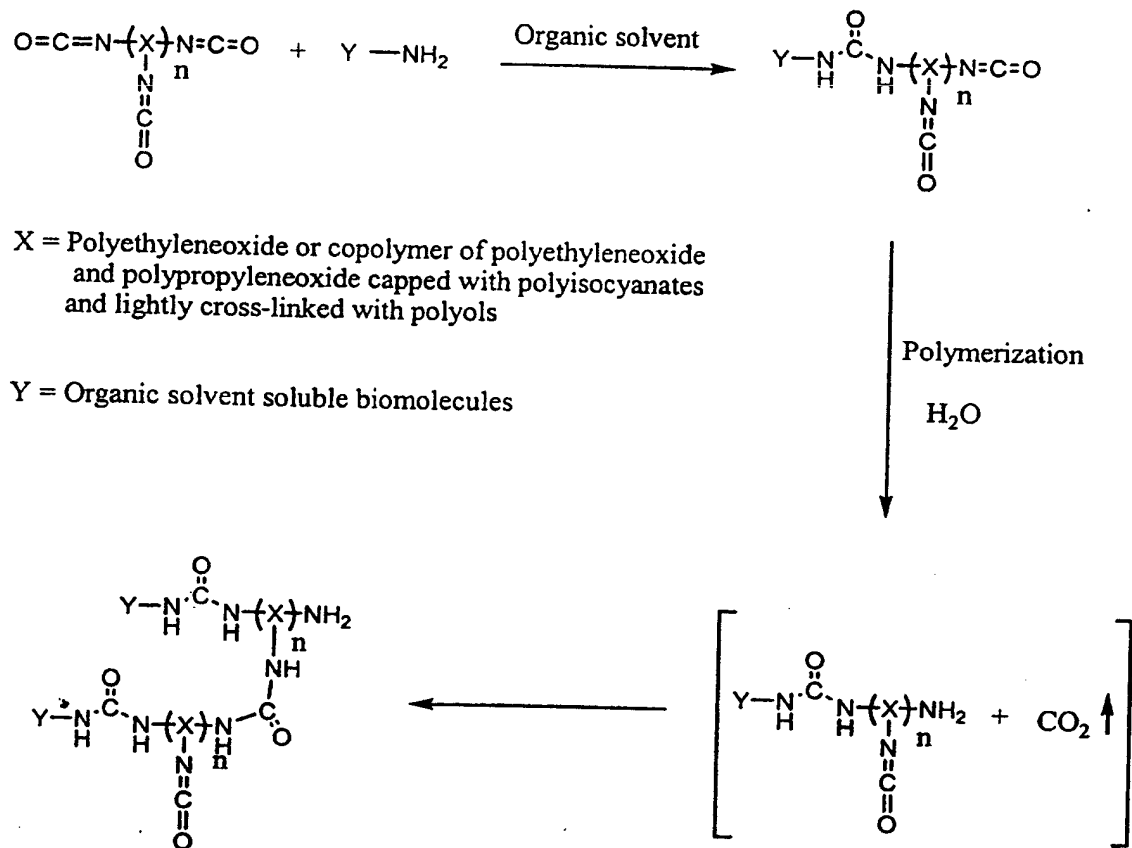
(g) contacting the washed biochip with an analyte solution, containing a target biomolecule, under hybridization conditions;

(h) then washing the biochip from step (g) with a second washing buffer such that non-specifically bound and unbound target biomolecule is removed; and

(i) detecting the target biomolecule bound to the hydrogel chip.

37. A hybridization assay according to claim 36 wherein the polymerizing hydrogel prepolymer is dispensed to create at least one individual cell on the substrate having a thickness between about 30  $\mu\text{m}$  and about 50  $\mu\text{m}$  and wherein the analyte solution contains a target biomolecule having a fluorescent marker bound thereto.

1 / 2



Continuation of polymerization  
until all isocyanates are consumed.

FIG. 1

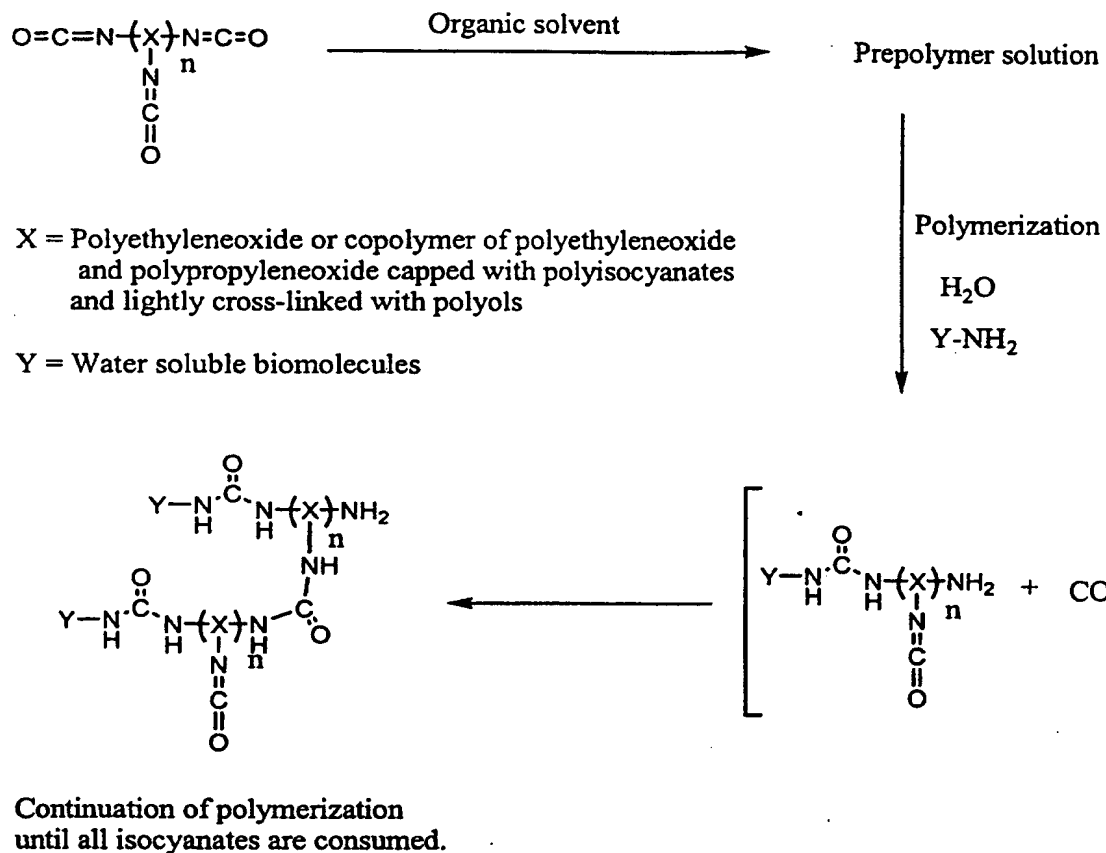


FIG. 2

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/11282

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q C07K C12N C08G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, EPO-Internal, COMPENDEX, INSPEC, BIOSIS, CHEM ABS Data, MEDLINE, SCISEARCH

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

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Date of the actual completion of the international search

21 September 2000

Date of mailing of the international search report

04/10/2000

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# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 00/11282

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